Class III phosphoinositide 3-kinase–Beclin1 complex mediates the amino acid-dependent regulation of autophagy in C_2C_{12} myotubes

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Increased proteolysis contributes to muscle atrophy that prevails in many diseases. Elucidating the signalling pathways responsible for this activation is of obvious clinical importance. Autophagy is a ubiquitous degradation process, induced by amino acid starvation, that delivers cytoplasmic components to lysosomes. Starvation markedly stimulates autophagy in myotubes, and the present studies investigate the mechanisms of this regulation. In C_2C_{12} myotubes incubated with serum growth factors, amino acid starvation stimulated autophagic proteolysis independently of p38 and p42/p44 mitogen-activated protein kinases, but in a PI3K (phosphoinositide 3-kinase)-dependent manner. Starvation, however, did not alter activities of class I and class II PI3Ks, and was not sufficient to affect major signalling proteins downstream from class I PI3K (glycogen synthase kinase, Akt/protein kinase B and protein S6). In contrast, starvation increased class III PI3K activity in whole-myotube extracts. In fact, this increase was most pronounced for a population of class III PI3K that co-immunoprecipitated with Beclin1/Apg6 protein, a major determinant in the initiation of autophagy. Stimulation of proteolysis was reproduced by feeding myotubes with synthetic dipalmitoyl-PtdIns3*P*, the class III PI3K product. Conversely, protein transfection of anti-class III PI3K inhibitory antibody into starved myotubes inverted the induction of proteolysis. Therefore, independently of class I PI3K/Akt, protein S6 and mitogen-activated protein kinase pathways, amino acid starvation stimulates proteolysis in myotubes by regulating class III PI3K–Beclin1 autophagic complexes.

Key words: Apg protein, macroautophagy, muscle cell, phosphatidylinositol 3-kinase, proteolysis, signal transduction.

INTRODUCTION

Amino acids act as signals that regulate protein metabolism. *In vitro* and *in vivo* studies demonstrated that amino acids activate p70S6K (p70 ribosomal S6 kinase) and translation initiation factors in muscle, the consequence being the stimulation of protein synthesis [1]. In contrast, the regulation of protein degradation by amino acids has been poorly studied in muscle cells. Skeletal muscle contains three major proteolytic pathways, the Ca^{2+}-activated calpains, the ubiquitin proteasome-dependent system and the lysosomal pathway [2]. Cytoplasmic components can be delivered to the lysosome by microautophagy, chaperone-mediated autophagy and macroautophagy (hereafter referred to as autophagy) [3]. In mammalian cells [4], including differentiated muscle cells [5], lysosomal autophagy represents the major proteolytic pathway stimulated in response to nutrient limitation.

During the autophagic process, cytoplasmic constituents are sequestered by a pre-autophagosomal structure to generate a double membrane-bound vesicle termed the autophagosome [4]. The autophagosome then fuses with a lysosome to become an autolysosome, where sequestered components are degraded for recycling. Genetic screenings in yeast have identified 16 *APG* genes required for autophagy, and many Apg products have related homologous proteins in mammals [6]. Moreover, Apg encoding mRNAs are broadly distributed in human and mouse tissues, often being abundant in skeletal muscle [7,8]. Expression studies revealed that post-translational modifications of Apg proteins are important for autophagic sequestration [6]. They encompass two ubiquitin-like modification systems, which were also characterized in mammals: protein conjugation of Apg12 to Apg5 [7], and conjugation of LC3 (microtubule-associated protein light chain 3; mammalian Apg8) to membrane phospholipid(s) [9]. Autophagic sequestration also involves two other multi-protein components. The first complex contains a protein kinase Apg1 [10], and is not yet characterized in mammals. The second complex contains PI3K (phosphoinositide 3-kinase) of class III, its adapter p150 and Beclin1 (mammalian Vps34, Vps15 and Apg6, respectively) [11,12]. This autophagy-specific class III–PI3K complex appears to be essential to recruit the Apg12–Apg5 conjugate to the pre-autophagosomal structure [13]. Apg12–Apg5 conjugation is then required for the elongation of the isolation membrane and for the proper localization of conjugated LC3/Apg8 [14]. Finally, the activity of Apg1 appears to be important for the completion of the autophagosome [13].

The autophagic pathway is subject to complex regulation in mammals. Various signal-transduction proteins, including ERK1/2 (extracellular-signal-regulated kinases 1 and 2) [15] and p38 [16] MAPKs (mitogen-activated protein kinases), heterotrimeric G-proteins [15], PI3Ks [17], mTOR (mammalian target of rapamycin) and p70S6K [3], were implicated in distinct pathways for the regulation of autophagy. Notably, three of these transduction pathways appear to be regulated by nutrient availability. Firstly, amino acids may activate mTOR and its downstream target p70S6K. This regulation has been the subject of intense studies [18], and was recently shown to be mediated by the TSC (tuberous sclerosis) complex [19]. In most cell types, inhibition of mTOR stimulates autophagy, although amino acids do not systematically regulate the mTOR/p70S6K pathway [5,20].

Abbreviations used: E64d, (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; ERK, extracellular-signal-regulated kinase; GSK, glycogen synthase kinase; LC3, microtubule-associated protein light chain 3; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal S6 kinase; PI3K, phosphoinositide 3-kinase; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyrydil)1H-imidazole; TSC, tuberous sclerosis.

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Secondly, in colon cancer cells phosphorylation of ERK1/2 is sensitive to amino acids. In this pathway, activated ERK1/2 phosphorylates a G_{α}-interacting protein, which stimulates the GTPase activity of the $G_{\alpha i3}$ protein and thereby favours autophagic sequestration [15]. Thirdly in hepatocytes, some amino acids (Ala, Gln) cause cell swelling due to their Na^+-coupled transport across the cell membrane. By activating class I PI3K/p70S6K [21] or p38 [16] transduction pathway, swelling may then down-regulate autophagy.

The Apg protein(s) that are primarily targeted by these amino acid-dependent transduction pathways remain to be identified in mammals. In fact, the Apg machinery exhibits few modifications in response to starvation. Apg8/LC3 appears as the only Apg that is substantially induced under starvation conditions [6]. The lipidation of Apg8/LC3 is also stimulated by nutrient limitation [9], but the conjugation of Apg12–Apg5 is constitutive [6]. Whether Ulk1, a potential mammalian Apg1 homologue [8], exhibits regulated protein kinase activity is also not known at present. Finally, class III PI3K functions with Beclin1/Apg6 as a core complex of the autophagic system [11], is central for the initiation of sequestration [13], but its regulation by nutrient availability has not been documented so far.

In the present study we investigated the transduction pathways through which amino acid limitation mediates autophagic proteolysis in differentiated muscle cells. We report that amino acid limitation regulates proteolysis independently of classes I and II PI3Ks, of Akt and S6, and of the ERK1/2 and p38 MAPK pathways. In contrast, we provide corroborative evidence that the class III PI3K–Beclin1 complex plays a preponderant role in the autophagic response of muscle cells to amino acid limitation.

MATERIALS AND METHODS

C_2C_{12} myotubes cell culture

Adult mouse muscle satellite C_2C_{12} cells were from the American Type Culture Collection (Manassas, VA, U.S.A.), and were grown and differentiated into myotubes as described in [5]. To investigate the role of amino acids, myotubes were incubated in test medium which was Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture, or amino acid-free Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture, both supplemented with 2 % dialysed horse serum and 2 mM L-Met, and an inhibitor when indicated.

Immunoblotting

C_2C_{12} myotubes were lysed in ice-cold lysis buffer containing 20 mM Tris/HCl (pH 7.4), 130 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 % Triton X-100, 1 mM PMSF and 1 × protease and phosphatase inhibitor cocktail (Sigma-Aldrich, L'Isle-d'Abeau Chesnes, France), and were clarified at 12 000 ***g*** for 5 min. Aliquots (80 μg of protein) were resolved by SDS/12 %-PAGE, electrotransferred to Hybond™ membranes (Amersham Biosciences, Orsay, France), and probed with affinity-purified polyclonal anti-phospho-ERK1/2 (Tyr-204), anti-phospho-p38 (Tyr-182), anti-phospho-Akt (Ser-473; Santa Cruz Biotechnology, Tebu, Le Perray-en-Yvelines, France), anti-phospho-GSK (glycogen synthase kinase) 3α/β (Ser-21/-9; Cell Signalling, Ozyme, Montigny Le Bretonneux, France) antibodies or with a monoclonal anti-Beclin1 antibody (Transduction Laboratories, Interchim, Montlucon, France). The polyclonal anti-phospho-S6 antibody was a gift from Dr M. J. Birnbaum (University of Pennsylvania, Philadelphia, PA, U.S.A.). Primary antibodies were resolved using an appropriate peroxidase-conjugated secondary antibody and the ECL Plus™ system (Amersham Biosciences).

Protein degradation

Myotubes (24-well dishes) were labelled for 18 h with L-[^{35}S]Met (8 μCi/ml), rapidly washed and then incubated for 1 h in DMEM/F12 (0.5 ml/well) supplemented with 2 % (v/v) dialysed horse serum and 2 mM unlabelled L-Met. A second 1 h pre-chase was performed using the test medium. The degradation period was started (zero time) by replacing the medium with fresh test medium (0.75 ml/well), and aliquots (20 μl) of extracellular medium were collected at the indicated times. Intracellular protein degradation was measured as described previously [22], and rates (k_d) of proteolysis were evaluated by the equation describing a first-order decay: $A_t = A_0\exp(-k_d t)$, with $A_t = A_0 - S_t$, and where A_0 is the total radioactivity incorporated in cell proteins at zero time, A_t is the radioactivity remaining in cell proteins at time t and S_t is the acid-soluble radioactivity at time t.

Liposome formation and dipalmitoyl-PtdIns3*P* treatment of C_2C_{12} myotubes

Synthetic dipalmitoyl-PtdIns3*P* (0.1 mg/ml) and the carrier phosphatidylserine (0.1 mg/ml) were solubilized in chloroform/methanol (1:1, v/v), dried under N_2 and sonicated for 15 min in 20 mM Tris/HCl (pH 7.4), using a bath sonicator. Liposomes were added to [^{35}S]Met pre-labelled myotubes before measurement of protein breakdown.

Pep1-mediated delivery of antibodies into myotubes

Pep1–antibody complexes were formed by incubating 0.6 μg of anti-class III PI3K, anti-Beclin1 or non-immune serum, with 4 μg of presonicated Pep1 (Chariot™; Active Motif, Rixensart, Belgium) in PBS (pH 7.4) for 30 min at 25 °C. [^{35}S]Met pre-labelled myotubes were overlaid with the pre-formed complexes according to the supplier's recommendations. The delivery of Pep1–antibody complexes was for 3 h at 37 °C (during the pre-chase periods), before measurement of protein degradation.

Analysis of class I PI3K activity

Myotubes were washed with ice-cold buffer A (50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF and 2 mM Na_3VO_4) and extracted for 15 min at 4 °C with buffer A supplement with 0.5 mM PMSF, 1 % Nonidet P-40 and 1 × inhibitor cocktail. After 10 min centrifugation at 10 000 ***g***, clarified supernatants were immunoprecipitated for 2 h at 4 °C with affinity-purified polyclonal anti-p85 class I PI3K (Santa Cruz Biotechnology) pre-absorbed on Protein A–Sepharose. The pellets were washed twice with each of the following buffers: (i) PBS (pH 7.4) and 1 % Nonidet P-40; (ii) 100 mM Tris/HCl (pH 7.4) and 0.5 M LiCl; and (iii) 10 mM Tris/HCl (pH 7.4), 100 mM NaCl and 1 mM EDTA. Immobilized proteins were resuspended in 30 μl of buffer 1 (20 mM Hepes, pH 7.5, 0.4 mM EGTA and 0.4 mM Na_2HPO_4), and supplemented with 10 μl of presonicated PtdIns (1 μg/μl) in 5 mM Hepes (pH 7.5).

Class I PI3K lipid kinase assay was started by adding 10 μl of buffer 1 supplemented with 1 μCi/μl [γ-^{32}P]ATP, 250 μM ATP-Na_2 and 50 mM $MgCl_2$. After 20 min incubation at 37 °C, the reactions were stopped by adding 15 μl of 4 M HCl and 130 μl of chloroform/methanol (1:1, v/v). Extracted lipids were separated

on Silica Gel 60A (Merck VWR, Limonest, France) with chloroform/methanol/4.3 M ammonia (9:7:2, by vol.). Lipids were identified by comparison with unlabelled phosphoinositide after exposure to iodine vapour. Radiolabelled PtdIns3*P* was quantified by PhosphorImager scanning (Molecular Dynamics, Bondoufle, France).

Analysis of class II PI3K activity

Cells were disrupted by successive aspirations through a 26-gauge needle into 20 mM Tris/HCl (pH 7.4), 125 mM NaCl, 0.2 mM EGTA, 0.2 mM EDTA, 1 mM Na_3VO_4 and 0.1 × inhibitor cocktail. This low concentration of inhibitor cocktail was used to reduce DMSO content (0.1 % final) and avoid dissolution of cell membranes. The lysate was centrifuged for 10 min at 2500 ***g*** and the resulting supernatant centrifuged for 2 h at 100 000 ***g***. The 100 000 ***g*** pellet was resuspended in reaction buffer 2 (20 mM Tris/HCl, pH 7.4, 125 mM NaCl, 0.2 mM EGTA and 0.2 mM EDTA), and 30 μl aliquots were supplemented with 10 μl of sonicated PtdIns (1 μg/μl) and pre-incubated for 10 min. The reaction was started by adding 10 μl of 20 mM Tris/HCl (pH 7.4), 125 mM NaCl, 1 μCi/μl [γ-^{32}P]ATP, 250 μM ATP-Na_2 and and 25 mM of $CaCl_2$. After 15 min incubation at 37 °C, reaction products were analysed as described above.

Analysis of class III PI3K activity

Class III PI3K activity was initially assessed in the supernatants obtained after immunoprecipitation of class I PI3K, as described in [17], using $MnCl_2$ as the preferred cation and 0.3 mM adenosine to inhibit phosphoinositide 4-kinase. Alternatively, C_2C_{12} myotubes were extracted for 15 min at 4 °C with 20 mM Tris/HCl (pH 7.4), 130 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 × inhibitor cocktail and 1 % Triton X-100. After 10 min centrifugation at 10 000 ***g***, clarified supernatants were immunoprecipitated for 2 h at 4 °C with 2 or 50 μg/ml anti-class III PI3K [23] or with 2 μg/ml anti-Beclin1 antibody pre-absorbed on Protein A–Sepharose. The pellets were washed twice with extraction buffer, and once with reaction buffer 3 (20 mM Tris/HCl, pH 7.4, 130 mM NaCl and 0.2 mM EDTA). The immobilized proteins were suspended in 30 μl of reaction buffer 3, supplemented with 10 μl of sonicated PtdIns (1 μg/μl) and pre-incubated for 10 min at room temperature. Reactions were started by adding 10 μl of reaction buffer 3 supplemented with 1 μCi/μl [γ-^{32}P]ATP, 250 μM ATP-Na_2 and 25 mM $MnCl_2$. After 15 min incubation at 37 °C, phospholipid products were analysed as described above.

General methods

Activities of lysosomal cathepsins B [24] and D [25] were measured using benzyloxycarbonyl-L-arginyl-L-arginine 4-methyl-coumaryl-7-amide and (7-methoxycoumarin-4-yl)acetyl-glycyl-L-lysyl-L-prolyl-L-isoleucyl-L-leucyl-L-phenylalanyl-L-phenylalanyl-L-arginyl-L-leucyl-N^{ε}-(2,4-dinitrophenyl)-L-lysyl-D-arginine amide (Peptide Institute, Osaka, Japan), respectively, as substrates. Benzyloxycarbonyl-L-phenylalanyl-L-arginine 4-methyl-coumaryl-7-amide (Peptide Institute) was used at 10 μM [22] to assay lysosomal cathepsin L-like endopeptidases (cathepsins L, S, O and Z). In C_2C_{12} myotubes, 5 μM pepstatin-A selectively inhibits cathepsin D and 10 μM E64d [(2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester] inhibits the cysteine endopeptidases (cathepsins B and L-like). Autophagic sequestration was measured as described in [26], except that pepstatin-A and E64d were used to inhibit lysosomal cathepsins. Protein concentration and DNA content were measured as described previously [5].

RESULTS

ERK1/2 and p38 MAPK are not implicated in the amino acid-dependent regulation of myotube proteolysis

Amino acid deprivation rapidly and strongly increased intracellular protein degradation in differentiated C_2C_{12} myotubes (Figure 1A). This stimulation of proteolysis was prevented in the presence of 10 μM E64d and 5 μM pepstatin-A, which inhibited the major lysosomal endopeptidases, cathepsins D, B and L-like (Figure 1B). This lysosomal-dependent increase in proteolysis was not associated with any modification of lysosomal cathepsin D, B or L-like activities (Figure 1B), but was related to a stimulation of autophagic sequestration (Figure 1C), and was prevented by 3-methyladenine [5], an inhibitor of autophagy.

Recent studies suggest the implication of ERK1/2 [15] and p38 [16] MAPK in the regulation of autophagy. In order to explore the role of the ERK1/2 transduction pathway in myotube autophagy, we used the compound PD98059 (2′-amino-3′-methoxyflavone), which binds the inactive form of MEK (MAPK/ERK kinase) and prevents ERK1/2 activation in C_2C_{12} myotubes ([27] and results not shown). In myotubes incubated in the presence of serum, inhibition of the ERK1/2 pathway did not alter basal rates of proteolysis and did not prevent the stimulation of proteolysis by amino acid withdrawal (Figure 2A). As monitored by anti-peptide immunoblot (Figure 2C), amino acid deprivation also did not affect the phosphorylation status of ERK1/2 in C_2C_{12} myotubes.

Phosphorylation of the activation loop of p38 was stimulated transiently (at 5 min) in amino acid-free medium, but was not altered at later times (Figure 2C). To investigate an eventual implication of p38 in the regulation of proteolysis by amino acid deprivation, the compound SB203580 [4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyrydil)1H-imidazole] was used to inhibit this transduction pathway [28]. Inhibition of p38 decreased rates of proteolysis in C_2C_{12} myotubes incubated in complete and also amino acid-free medium. Furthermore, inhibition of the p38 cascade did not prevent the stimulation of proteolysis by amino acid depletion (Figure 2B). These data indicate that, in C_2C_{12} myotubes, ERK1/2 and p38 MAPK pathways do not play a significant role in the stimulation of intracellular proteolysis by amino acid withdrawal.

Wortmannin prevents amino acid-dependent regulation of myotube proteolysis

To determine if PI3K(s) are implicated in the amino acid-dependent regulation of protein breakdown, C_2C_{12} myotubes were incubated in the presence of various concentrations of wortmannin, which inhibits PI3Ks by covalently interacting with their active sites [29]. As shown in Figure 3(B), wortmannin slightly, though not significantly, reduced basal rates of proteolysis in myotubes incubated in complete medium. Importantly, inhibition of the PI3Ks by wortmannin rapidly abolished the acceleration of proteolysis generated by amino acids withdrawal (Figure 3A). The amino acid-dependent proteolysis was reduced in a dose-dependent manner (Figure 3B), and full inhibition was obtained with 7–10 nM of wortmannin, a concentration expected to selectively inhibit classes I, IIβ and III of PI3Ks [30].

These results suggest that PI3K(s) is (are) required for the regulation of protein breakdown by amino acids. In fact, PI3Ks are implicated in a variety of fundamental cellular processes,

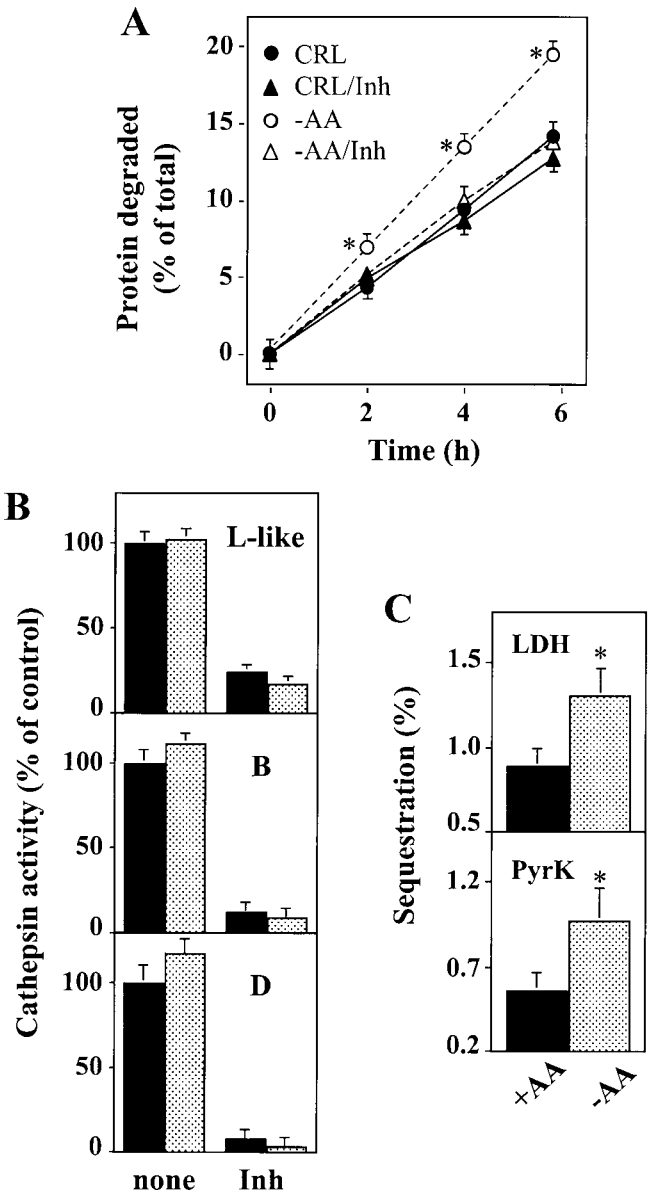

Figure 1 Amino acid withdrawal stimulates autophagic sequestration in C_2C_{12} myotubes

(**A**) [^{35}S]Met radiolabelled myotubes were incubated in control medium (CRL; closed symbols) or in medium without amino acids (−AA; open symbols), and supplemented with the inhibitors (Inh; triangles) 10 μM E64d and 5 μM pepstatin-A, or with vehicle alone (circles). Protein degradation was measured as described in the Materials and methods section. (**B**) Similar incubations were performed in medium with (black bars) or without (stippled bars) amino acids, but without radioactive labelling to measure lysosomal-autophagosomal activities of cathepsin L-like, cathepsin B and cathepsin D endopeptidases. (**C**) Autophagic sequestration of lactate dehydrogenase (LDH) and pyruvate kinase (PyrK) was measured as described in [26], but using E64d and pepstatin-A to inhibit lysosomal proteolysis. Values are means ± S.E.M. from four to ten different experiments. *$P < 0.01$ indicates statistically significant differences from the group with amino acids.

including cell survival and proliferation, cell motility and adhesion, cytoskeletal rearrangement and vesicle trafficking [31]. A simple explanation for our observations could be that inhibition of PI3Ks alters a spectrum of fundamental cellular activities, some of which are critical to maintain a functional autophagic machinery. If PI3K(s) directly mediate(s) the control of proteolysis by amino acids, their lipid-kinase activity should increase in response to amino acid depletion. Furthermore, this increase in PI3K(s) activity should occur before the induction of lysosomal proteolysis. In C_2C_{12} myotubes, amino acid depletion accelerates proteolysis by stimulating lysosomal autophagy as early as 30 min after medium change [5]. We then carried out further studies to specify which classes of PI3K are altered in C_2C_{12} myotubes shortly after amino acid depletion.

Amino acid depletion does not affect class I and class II PI3K activities in C_2C_{12} myotubes

Class I PI3Ks are heterodimers made up of a catalytic subunit and an adapter subunit. While class I_B is mostly restricted to myeloid cells [32], class I_A PI3Ks exhibit a ubiquitous distribution. Class I_A adapter subunits (p85α, p85β) associate with the different isoforms of p110 catalytic subunits [33]. An antibody directed towards p85 adapter subunits was used to immunoprecipitate total cellular lysates and to assess class I_A PI3K total lipid kinase activity. Time-course experiments performed with C_2C_{12} myotubes revealed that amino acid starvation did not alter class I_A PI3K activity in p85 immunoprecipitates (Figure 4A). PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (e.g. *in vivo* products of class I_A PI3K activity) mediate the stimulation of Akt, which activates multiple downstream signalling pathways. In agreement with the lack of variation of class I_A PI3K activity, amino acid deprivation also did not affect phosphorylation of Akt (Figure 4B). To monitor independently the effect of amino acid deprivation on Akt downstream signalling [27], we investigated phosphorylation of GSK3 and S6 protein. While GSK3 is a direct target of Akt, S6 phosphorylation implicates the inactivation of the TSC1/2 inhibitory complex and the subsequent stimulation of mTOR and p70S6K [34]. As shown in Figure 4(B), amino acid withdrawal did not modify the phosphorylation of GSK3 and S6.

The supernatants resulting from p85-immunoprecipitation nonetheless revealed PI 3-kinase activity, that was stimulated 2–3-fold at initial time points after amino acid deprivation (Figure 4A). This lipid kinase activity may correspond to class II [30] or III of PI3K [17]. Class II PI3Ks are unique in being able to use Ca^{2+} for their kinase activity [35]. Further experiments indicated that Ca^{2+}-dependent class II PI3K activity was low in C_2C_{12} myotubes and was not altered by amino acid depletion (results not shown).

Amino acid depletion increases class III PI3K activity in C_2C_{12} myotubes

To assess whether class III PI3K is regulated by amino acid withdrawal, myotube extracts were immunoprecipitated with low concentrations of an antibody raised against this enzyme [23]. Measurement of Mn^{2+}-dependent PtdIns kinase in these immunoprecipitates indicated that amino acid depletion increased class III PI3K activity in C_2C_{12} myotubes (Figure 5A, left-hand lanes). Stimulation of PtdIns kinase activity, however, was only detected when low concentrations of antibodies were used to immunoprecipitate class III PI3K. Notably, this antibody at high concentrations is known to inhibit specifically class III PI3K activity [23,36] (Figure 5A, right-hand lanes). Alternative experiments were then required to confirm that, in response to amino acid depletion, the accelerated proteolysis is associated with an activation of class III PI3K in C_2C_{12} myotubes.

Amino acid depletion increases class III PI3K activity complexed with Beclin1

Class III PI3K has been implicated in a wide range of cellular phenomena, including autophagy [17], phagocytosis [36] and

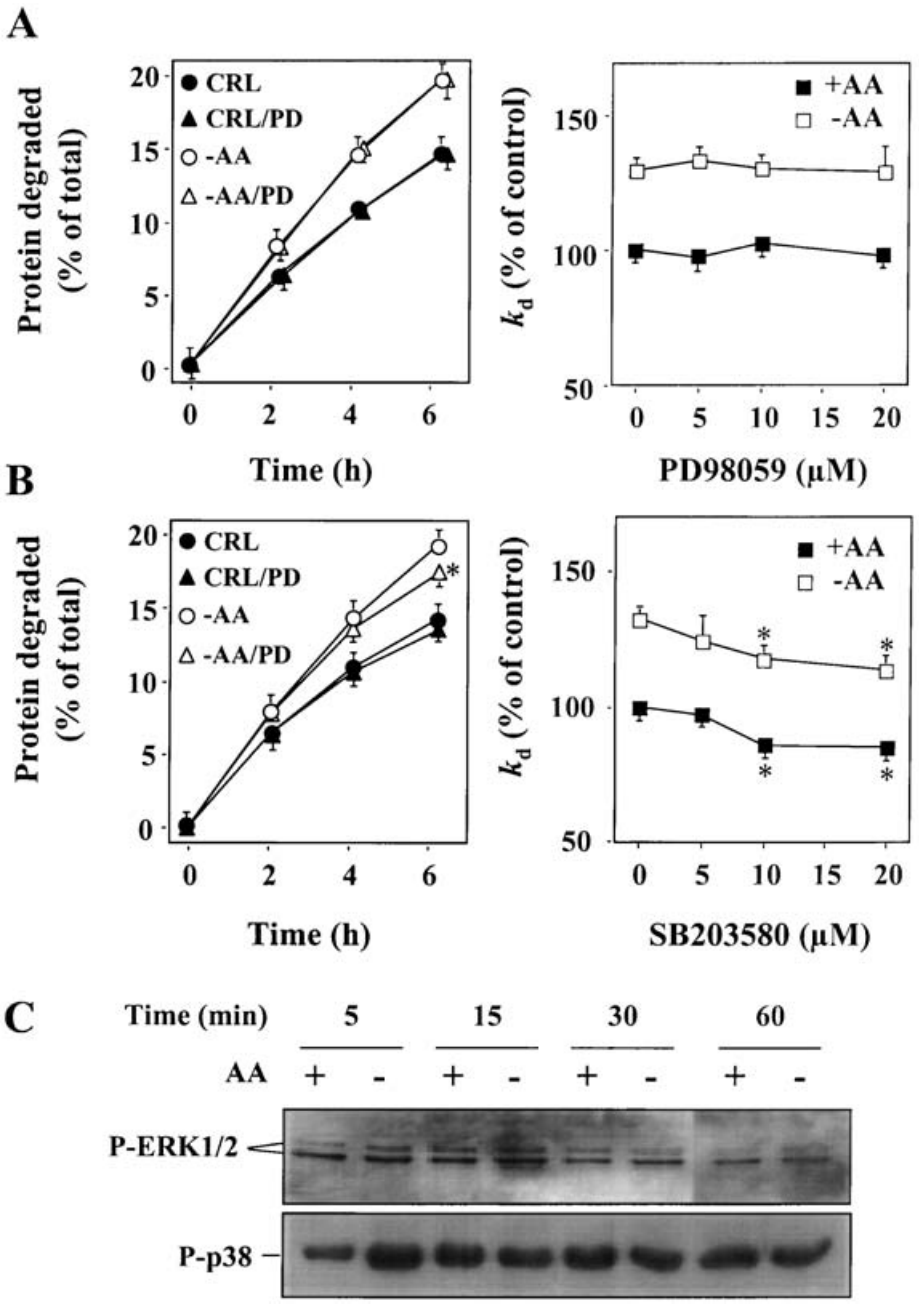

Figure 2 Amino acid withdrawal stimulates proteolysis independently of ERK1/2 and p38 MAPK in C_2C_{12} myotubes

[^{35}S]Met radiolabelled myotubes, were incubated in medium with (CRL; closed symbols) or without amino acids (−AA; open symbols), and supplemented with vehicle alone (circles) or with (PD; triangles); PD98059 (**A**) or SB203580 (**B**). For time-course studies (left-hand panels), PD98059 and SB203580 were used at 10 μM; for dose-response studies (right-hand panels), rates (k_d) of protein degradation were estimated over a 6 h incubation period, as described in the Materials and methods section. Values are means ± S.D. ($n = 6$), and $^*P < 0.01$ indicates statistically significant differences from the group without inhibitor. (**C**) Myotubes were incubated for the indicated times in medium with (+) or without (−) amino acids (AA), and cell lysates were subjected to immunoblot analysis of phospho-ERK1/2 or phospho-p38.

post-endocytic sorting of receptors [23]. Subcellular distribution of class III PI3K to nascent autophagosomes is likely to be required for this lipid kinase to specifically control autophagy. In yeast and HeLa cells [11,12], Vps34/class III PI3K forms macromolecular complexes with the autophagic protein Apg6/Beclin1. We thus hypothesized that in C_2C_{12} myotubes a population of class III PI3K forms complexes with Beclin1, and that these complexes may be favoured when autophagy is stimulated by amino acid depletion.

To assess this hypothesis, total cellular extracts were immunoprecipitated with the anti-class III PI3K and precipitants immunoblotted with anti-Beclin1. In C_2C_{12} myotubes, 61 kDa Beclin1 co-immunoprecipitated with class III PI3K, and amino acid depletion tended to increase Beclin1 immunoprecipitated

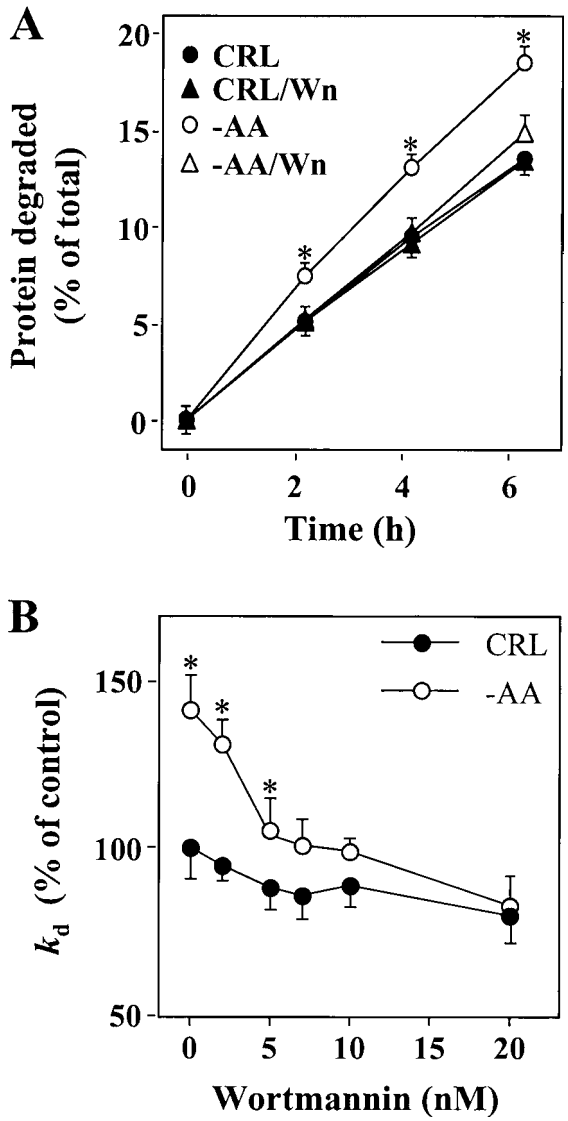

Figure 3 Amino acid-dependent stimulation of proteolysis is prevented by wortmannin in C_2C_{12} myotubes

(**A**) [^{35}S]Met radiolabelled myotubes were incubated in medium with (CRL; closed symbols), or without amino acids (−AA; open symbols), and supplemented (triangles) or not (circles) with 20 nM wortmannin (Wn). (**B**) Dose–response of wortmannin on proteolysis in myotubes incubated with (closed circles) or without (open circles) amino acids. Rates (k_d) of protein degradation were estimated over a 6 h incubation period. Values are means ± S.D. ($n = 6$), and $^*P < 0.01$ indicates statistically significant differences from the group with amino acids.

with class III PI3K (Figure 5B, right-hand lanes), without affecting total levels of Beclin1 in whole-cell extracts (Figure 5B, left-hand lanes). Reciprocally, immunoprecipitations were performed with an anti-Beclin1 antibody to confirm that class III PI3K exists in macrocomplexes with Beclin-1. Interestingly, amino acid withdrawal strongly increased PI3K activity measured in Beclin1 immunoprecipitates (Figure 5C). This PI3K activity was inhibited when myotubes were pre-treated by the PI3K inhibitor wortmannin (Figure 5D). Time-course studies indicated low basal activity in control myotubes (Figure 5E, 5 min), which may suggest limited autophagy in cells maintained in low-serum medium. However, time-course studies emphasized that amino acid limitation induced a pronounced and transitory increase [7.6±2.2-fold ($n = 6$) at 10 min] of the class III PI3K activity complexed to Beclin1 in C_2C_{12} myotubes (Figure 5E).

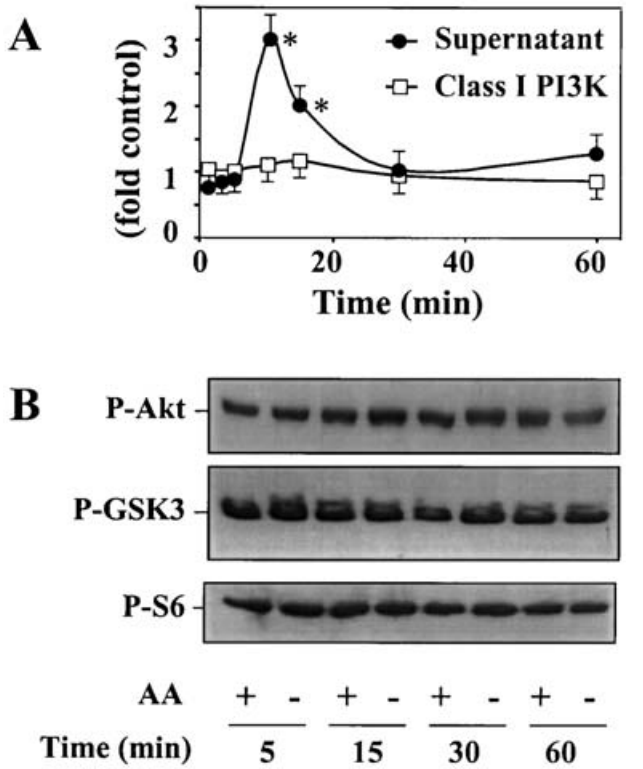

Figure 4 Amino acid depletion does not affect class I PI3K pathway in C_2C_{12} myotubes

(**A**) Myotubes were incubated in medium containing or not amino acids, and lysates were immunoprecipitated with anti-p85 class I PI3K antibody. PI3K activity was measured in the p85-immunoprecipitates (□) and in the supernatants (●). The charts show relative variations of PI3K activity in myotubes incubated without amino acids; values are expressed as a multiple of the value in complete medium, and are means ± S.E.M. from three to six different experiments. (**B**) Myotubes were incubated for the indicated times in medium with (+) or without (−) amino acids (AA), and cell lysates were subjected to immunoblot analysis of phospho-Akt, phospho-GSK3 or phospho-S6.

The product of class III PI3K activity stimulates proteolysis in C_2C_{12} myotubes

In vivo class III PI3K phosphorylates only PtdIns, while the class I preferred substrate is PtdIns(4,5)P_2 [32]. If class III PI3K regulates proteolysis in myotubes, the product of its lipid kinase activity, PtdIns3*P*, should be sufficient to mimic this effect. Previous studies have indicated that synthetic phosphoinositide sonicated to form micelles can be added to cultured cells [17,37]. These micelles fuse with the cell membrane, and exogenous lipids are taken up into the cell and are biologically active. Micelles of synthetic dipalmitoyl-PtdIns3*P* were then supplied to myotubes incubated in complete medium. In accordance with an implication of class III PI3K in the control of autophagy, dipalmitoyl-PtdIns3*P* stimulated proteolysis in a dose-dependent manner and in parallel decreased cellular proteins without affecting total DNA content (Figure 6A).

Transfection of inhibitory anti-class III PI3K reduces proteolysis in starved myotubes

If class III PI3K is implicated in the regulation of proteolysis by amino acids, selective inhibition of its lipid kinase activity should reduce the accelerated proteolysis in starved myotubes. To assess this proposal, we used Pep1 to transfect inhibitory

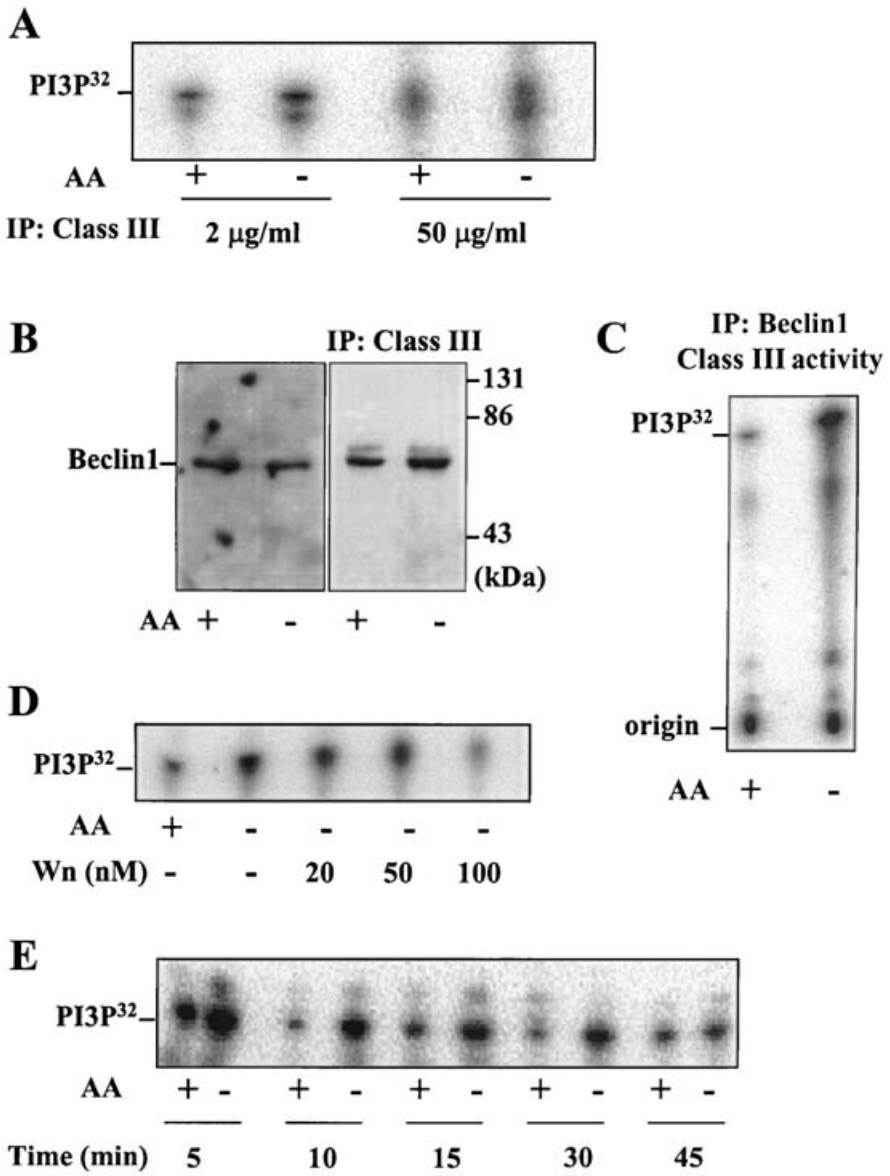

Figure 5 Amino acid withdrawal increases class III PI3K activity complexed with Beclin1 in C_2C_{12} myotubes

Myotubes were incubated for 15 min with (+) or without (−) amino acids (AA) and: (**A**) cell extracts were immunoprecipitated with low (2 μg/ml) or high (50 μg/ml) concentrations of anti-class III PI3K, and precipitants used to assay PI3K activity; (**B**) whole-cell lysates (left-hand panel) or lysates immunoprecipitated with anti-class III PI3K (right-hand panel) were analysed by Western blotting using a monoclonal anti-Beclin1 antibody; (**C**) alternatively, cell lysates were immunoprecipitated with anti-Beclin1 antibody and precipitants used for measurement of class III PI3K activity. (**D**) Cells were pre-treated for 30 min with 0–100 nM wortmannin, and incubated for 15 min with (+) or without (−) amino acids, and wortmannin when indicated; cell lysates were then immunoprecipitated with Beclin1 and precipitants used to assay class III PI3K activity. (**E**) Time-course study of class III PI3K activity co-immunoprecipitated with Beclin1 in C_2C_{12} myotubes incubated in medium with (+) or without (−) amino acids.

anti-class III PI3K. Pep1 is a non-toxic peptide which forms non-covalent complexes with proteins and delivers them into living cells in a fully biologically active form. In particular, Pep1 delivers antibodies into cells, while preserving their ability to recognize antigens within cells [38]. In C_2C_{12} myotubes, delivery of complexes of Pep1 with an irrelevant antibody did not alter rates of proteolysis (Figure 6B). Anti-Beclin1 antibody, when co-immunoprecipitated with class III PI3K, does not inhibit its PtdIns kinase activity (see Figure 5E). Accordingly, Pep1-mediated transfection of anti-Beclin1 also did not affect rates of proteolysis in C_2C_{12} myotubes (Figure 6B). In contrast, anti-class III PI3K antibody inhibits class III PI3K activity *in vitro* [23] (Figure 5A) and also *in situ* when microinjected into cultured cells [36]. In accordance with a role of class III PI3K in the regulation of autophagy by amino acids, Pep1-mediated delivery of anti-class III PI3K antibody into starved myotubes significantly reduced rates of protein degradation (Figure 6B).

DISCUSSION

Amino acids are key regulators of autophagic proteolysis, and our results provide new insights into the basic mechanisms of this regulation. Altogether our observations strongly support the proposal that amino acid withdrawal stimulates class III PI3K complexed to the autophagic protein Beclin1 and thereby locally

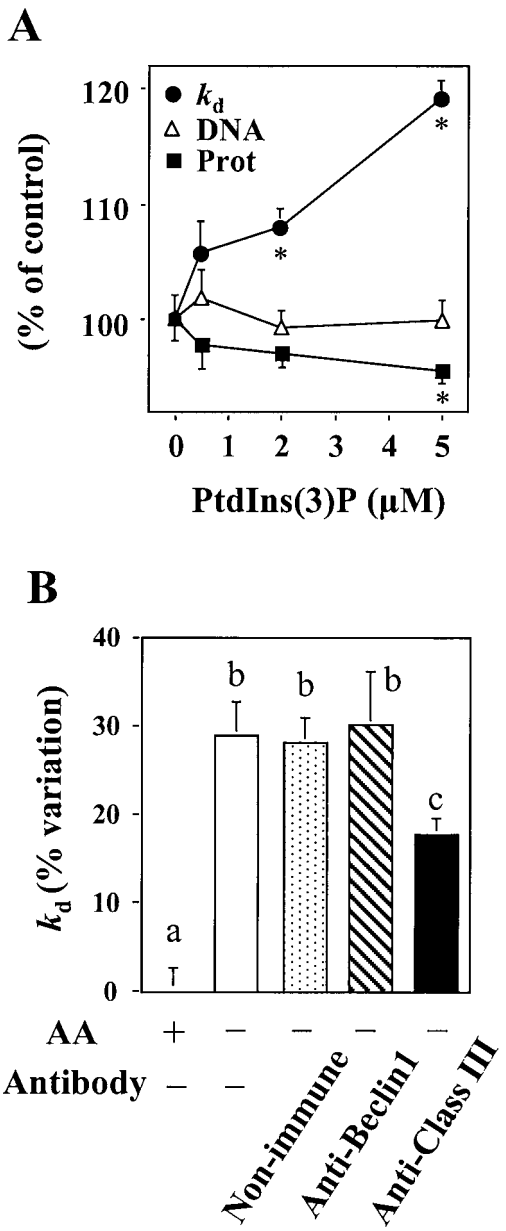

Figure 6 Endogenous class III PI3K regulates proteolysis in differentiated muscle cells

(**A**) Dose response of dipalmitoyl-PtdIns3*P* on rates of intracellular proteolysis (●), and on DNA (△) and protein content (■), after 6 h incubation. Values are means ± S.D. ($n = 6$), and $^*P < 0.01$ indicates statistically significant differences from the group without PtdIns3*P*. (**B**) [^{35}S]Met radiolabelled myotubes were overlaid for 3 h with the indicated antibody-Pep1 pre-formed complexes. Myotubes were then incubated in medium with (+) or without (−) amino acids (AA) and rates of protein degradation were measured. Values are means ± S.E.M. ($n = 3$), and are significantly different ($P < 0.01$) when they do not share a common letter (a, b or c).

increases PtdIns3*P*, which in turn stimulates autophagic proteolysis in C_2C_{12} myotubes.

Three major distinct MAPK pathways are described in mammalian cells, and two of them (ERK1/2 and p38) are reported to be important regulators of cellular autophagy. In colon cancer HT-29 cells, ERK1/2 are not only sensitive to amino acids but are also part of a signalling pathway that controls autophagy [15]. The response of ERK1/2 to amino acids, however, varies among cell types. Amino acids do not alter ERK1/2 phosphorylation in hepatocytes [39], in L6 muscle cells [40] and in C_2C_{12} myotubes (the present study). Moreover, in the present study, selective inhibition of ERK1/2 did not affect basal or stimulated proteolysis. In hepatocytes, cellular hydration state is reported to modulate autophagy via the p38 transduction pathway [16]. In C_2C_{12} myotubes, selective inhibition of p38 decreased proteolysis, but this occurred independently of amino acid sufficiency. Therefore, cell-type specificity and/or differentiation probably play an important role in the response of MAPKs to extracellular amino acids, and these pathways do not account for the regulation of autophagy by amino acids in differentiated muscle cells.

In contrast to our observations with MAPK inhibitors, wortmannin prevented the stimulation of myotube proteolysis generated by amino acid withdrawal, which suggested the implication of PI3K(s) in this regulation. The concentration dependence for wortmannin inhibition was similar to that of classes I, IIβ and III of PI3K [30], but lower than the concentration required for maximal inhibition of mTOR auto-kinase activity [41]. Previous studies performed in colon carcinoma HT-29 cells emphasized that both class I and III PI3Ks regulate autophagic sequestration, although with opposite effects [17]. In the present experiment, the simultaneous blockade of inhibitory class I and stimulatory class III did not allow a straightforward interpretation of experiments using PI3K inhibitors. We thus assessed all three classes of wortmannin-sensitive PI3Ks and our study unequivocally demonstrates that amino acid depletion stimulates class III PI3K to up-regulate autophagy in C_2C_{12} myotubes.

Amino acid deprivation altered neither Ca^{2+}-dependent activity of class II PI3K in microsomes, nor class I PI3K lipid kinase activity in p85 immunoprecipitates. Class I PI3K products recruit Akt to the plasma membrane, which allows its activating phosphorylation [32]. Accordingly in C_2C_{12} myotubes, amino acid deprivation did not alter Akt phosphorylation, and did not modify signalling proteins (GSK3, S6) downstream from Akt. These observations are in agreement with previous studies performed in other cell lines, which showed that amino acids rarely affect class I PI3K and Akt [18]. Although amino acids do not regulate class I PI3K and Akt, they usually regulate p70S6K and its downstream target S6 [18]. The mechanistic basis of this phenomenon was recently found to implicate an Akt-independent regulation by amino acids of mTOR via the TSC1/2 complex [19]. The regulation of mTOR/p70S6K/S6 is considered central to the control of autophagy and protein synthesis [18], and is functional in C_2C_{12} myotubes, as rapamycin-inhibition of mTOR stimulates autophagy [5]. However, such amino acid-dependent and Akt-independent regulation of S6 ([5]; Figure 4B) and p70S6K (results not shown) did not occur in C_2C_{12} myotubes. A similar lack of p70S6K regulation after Leu deprivation was found in L6 myoblasts incubated in the presence of insulin [20]. In fact in several cell lines, mTOR/p70S6K/S6 pathway was shown to be sensitive to a wide variety of inputs that include serum, differentiation, osmotic stress, mitochondrial dysfunction as well as nutrients [42]. In our studies, the presence of serum, the cell type and/or the state of differentiation are likely to account for the lack of regulation of this pathway.

Although amino acid withdrawal, on its own, was not sufficient to alter ERK1/2 and p38 MAPKs, class I PI3K/Akt, and mTOR/S6 pathways, it nevertheless stimulated autophagy in C_2C_{12} myotubes. The present investigations demonstrate that class III PI3K mediates the amino acid-dependent regulation of autophagy in myotubes. Firstly, several of our observations emphasize that amino acid deprivation stimulates class III PI3K. Notably, starvation increased a wortmannin-sensitive PI3K activity (i) in

extracts depleted of class I PI3K by p85 immunoprecipitation (Figure 4A), (ii) in immunoprecipitates generated with low concentrations of anti-class III PI3K (Figure 5A), and (iii) in class III PI3K–Beclin1 complexes immunoprecipitated with anti-Beclin1 (Figure 5E).

Secondly, class III PI3K is the only PI3K producing PtdIns3*P* *in vivo*, and this product supplied as micelles to myotubes mimicked the stimulation of proteolysis generated by amino acid depletion. A similar stimulation of autophagic proteolysis was previously reported with PtdIns3*P* in HT-29 carcinoma cells, although the regulation of class III PI3K by amino acids was not investigated [17]. Thirdly, inhibition of endogenous class III PI3K reduced proteolysis in starved myotubes, which constitutes additional evidence that amino acid-dependent autophagy requires active class III PI3K. Pep1-mediated delivery of the inhibitory antibody, however, did not totally reverse the stimulated proteolysis, and this may be attributed to an incomplete inhibition of class III PI3K–Beclin1 *in vivo*, or alternatively, to the implication of other yet unknown pathway(s).

In vivo levels of PtdIns3*P* are reasonably constant in mammalian cells [43]. In signalling, PtdIns3*P* is believed to act locally, and through interactions with different effectors to regulate specific functions. In agreement with this notion, the stimulation of class III PI3K by amino acid deprivation was low in whole myotube extracts, but elevated in Beclin1 co-immunoprecipitates. Therefore, the subcellular compartmentalization of class III PI3K is probably critical for the regulation of autophagy. Hence, stimulation of class III PI3K by amino acid deprivation would not be expected strongly to alter global PtdIns3*P* concentration, but rather to increase locally PtdIns3*P* levels in pre-autophagosomal membranes.

The mechanism whereby amino acids regulate lipid kinase activity of class III PI3K remains unknown. Amino acid deprivation may stimulate association of class III PI3K with Beclin1 and/or the lipid kinase activity of class III PI3K pre-existing in Beclin1 complexes. The lipid kinase activity of class III PI3K is known to be stimulated by its association with its adapter p150 [23], and also by provision of its substrate PtdIns by the PtdIns-transfer protein [44]. Both class III PI3K and its adapter p150 appear to be phosphorylated [44] and these phosphorylations may regulate class III PI3K lipid kinase activity and/or association with Beclin1 complexes. The resulting production of PtdIns3*P* at specific intracellular locales may generate significant asymmetries and drive membrane curvature of pre-autophagosomes. Alternatively, PtdIns3*P* may be converted into higher-order polyphosphoinositides, that have known signalling functions. In fact, the main function of PtdIns3*P* is certainly to locally recruit PtdIns3*P*-binding proteins implicated in autophagic sequestration. PtdIns3*P* specifically interacts with a protein domain designated FYVE [43], which functions in the regulation of endosomal trafficking, but which is not yet described for autophagic sequestration. Whatever the precise mechanism is, the regulation of class III PI3K–Beclin1 complex is probably critical for autophagic sequestration, as this complex appears to be essential for the recruitment of Apg conjugates to the pre-autophagosomal structure [13].

The regulation of proteolysis in muscle cells is important in adaptations of the organism to a variety of stressful conditions. In fact, lysosomal cathepsin L was recently identified as a differential marker for muscle atrophy [45]. Defects in autophagy have serious consequences and have been linked to myopathies [46], cardiomyopathies [47], and also to neurodegenerative diseases [48], cancer [49] and aging [50]. Our investigation, which high-lights a novel mechanism for the regulation of autophagy, therefore has wide therapeutic relevance.

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REFERENCES

1 Proud, C. G. (2002) Regulation of mammalian translation factors by nutrients. Eur. J. Biochem. **269**, 5338–5349
2 Taillandier, D., Aurousseau, E., Meynial-Denis, D., Bechet, D., Ferrara, M., Cottin, P., Ducastaing, A., Bigard, X., Guezennec, C. Y., Schmid, H. P. and Attaix, D. (1996) Coordinate activation of lysosomal, Ca^{2+}-activated and ATP-ubiquitin dependent proteinases in the unweighted rat soleus muscle. Biochem. J. **316**, 65–72
3 Blommaart, E. F., Luiken, J. J. and Meijer, A. J. (1997) Autophagic proteolysis: control and specificity. Histochem. J. **29**, 365–385
4 Mortimore, G. E., Poso, A. R. and Lardeux, B. R. (1989) Mechanism and regulation of protein degradation in liver. Diabetes Metab. Rev. **5**, 49–70
5 Mordier, S., Deval, C., Bechet, D., Tassa, A. and Ferrara, M. (2000) Leucine limitation induces autophagy and activation of lysosome-dependent proteolysis in C2C12 myotubes through a mammalian target of rapamycin-independent signaling pathway. J. Biol. Chem. **275**, 29900–29906
6 Ohsumi, Y. (2001) Molecular dissection of autophagy: two ubiquitin-like systems. Nat. Rev. Mol. Cell Biol. **2**, 211–216
7 Mizushima, N., Sugita, H., Yoshimori, T. and Ohsumi, Y. (1998) A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. J. Biol. Chem. **273**, 33889–33892
8 Yan, J., Kuroyanagi, H., Kuroiwa, A., Matsuda, Y., Tokumitsu, H., Tomoda, T., Shirasawa, T. and Muramatsu, M. (1998) Identification of mouse ULK1, a novel protein kinase structurally related to *C. elegans* UNC-51. Biochem. Biophys. Res. Commun. **246**, 222–227
9 Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y. and Yoshimori, T. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. **19**, 5720–5728
10 Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M. and Ohsumi, Y. (2000) Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J. Cell Biol. **150**, 1507–1513
11 Kihara, A., Noda, T., Ishihara, N. and Ohsumi, Y. (2001) Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. J. Cell Biol. **152**, 519–530
12 Kihara, A., Kabeya, Y., Ohsumi, Y. and Yoshimori, T. (2001) Beclin–phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. EMBO Rep. **2**, 330–335
13 Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T. and Ohsumi, Y. (2001) The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. EMBO J. **20**, 5971–5981
14 Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y. and Yoshimori, T. (2001) Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J. Cell Biol. **152**, 657–668
15 Ogier-Denis, E., Pattingre, S., El Benna, J. and Codogno, P. (2000) Erk1/2-dependent phosphorylation of Galpha-interacting protein stimulates its GTPase accelerating activity and autophagy in human colon cancer cells. J. Biol. Chem. **275**, 39090–39095
16 vom Dahl, S., Dombrowski, F., Schmitt, M., Schliess, F., Pfeifer, U. and Haussinger, D. (2001) Cell hydration controls autophagosome formation in rat liver in a microtubule-dependent way downstream from p38MAPK activation. Biochem. J. **354**, 31–36
17 Petiot, A., Ogier-Denis, E., Blommaart, E. F., Meijer, A. J. and Codogno, P. (2000) Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. J. Biol. Chem. **275**, 992–998
18 van Sluijters, D. A., Dubbelhuis, P. F., Blommaart, E. F. and Meijer, A. J. (2000) Amino-acid-dependent signal transduction. Biochem. J. **351**, 545–550
19 Gao, X., Zhang, Y., Arrazola, P., Hino, O., Kobayashi, T., Yeung, R. S., Ru, B. and Pan, D. (2002) Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nat. Cell Biol. **4**, 699–704
20 Kimball, S. R., Horetsky, R. L. and Jefferson, L. S. (1998) Implication of eIF2B rather than eIF4E in the regulation of global protein synthesis by amino acids in L6 myoblasts. J. Biol. Chem. **273**, 30945–30953
21 Krause, U., Rider, M. H. and Hue, L. (1996) Protein kinase signaling pathway triggered by cell swelling and involved in the activation of glycogen synthase and acetyl-CoA carboxylase in isolated rat hepatocytes. J. Biol. Chem. **271**, 16668–16673

22 Tournu, C., Obled, A., Ferrara, M., Omura, S. and Bechet, D. (2001) Glucose regulates protein catabolism in ras-transformed fibroblasts through a lysosomal-dependent proteolytic pathway. Biochem. J. **357**, 255–261
23 Siddhanta, U., McIlroy, J., Shah, A., Zhang, Y. and Backer, J. M. (1998) Distinct roles for the p110alpha and hVPS34 phosphatidylinositol 3′-kinases in vesicular trafficking, regulation of the actin cytoskeleton, and mitogenesis. J. Cell Biol. **143**, 1647–1659
24 Bechet, D., Ferrara, M., Mordier, S., Roux, M. P., Deval, C. and Obled, A. (1991) Expression of lysosomal cathepsins during calf myoblast-myotube differentiation: characterization of a cDNA encoding bovine cathepsin B. J. Biol. Chem. **266**, 14104–14112
25 Yasuda, Y., Kageyama, T., Akamine, A., Shibata, M., Kominami, E., Uchiyama, Y. and Yamamoto, K. (1999) Characterization of new fluorogenic substrates for the rapid and sensitive assay of cathepsin E and cathepsin D. J. Biochem. **125**, 1137–1143
26 Kopitz, J., Kisen, G. O., Gordon, P. B., Bohley, P. and Seglen, P. O. (1990) Nonselective autophagy of cytosolic enzymes by isolated rat hepatocytes. J. Cell Biol. **111**, 941–953
27 Rommel, C., Bodine, S. C., Clarke, B. A., Rossman, R., Nunez, L., Stitt, T. N., Yancopoulos, G. D. and Glass, D. J. (2001) Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat. Cell Biol. **3**, 1009–1013
28 Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. **351**, 95–105
29 Djordjevic, S. and Driscoll, P. C. (2002) Structural insight into substrate specificity and regulatory mechanisms of phosphoinositide 3-kinases. Trends Biochem. Sci. **27**, 426–432
30 Fruman, D. A., Meyers, R. E. and Cantley, L. C. (1998) Phosphoinositide kinases. Annu. Rev. Biochem. **67**, 481–507
31 Rameh, L. E. and Cantley, L. C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function. J. Biol. Chem. **274**, 8347–8350
32 Vanhaesebroeck, B., Leevers, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J. and Waterfield, M. D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. Annu. Rev. Biochem. **70**, 535–602
33 Vanhaesebroeck, B., Welham, M. J., Kotani, K., Stein, R., Warne, P. H., Zvelebil, M. J., Higashi, K., Volinia, S., Downward, J. and Waterfield, M. D. (1997) P110delta, a novel phosphoinositide 3-kinase in leukocytes. Proc. Natl. Acad. Sci. U.S.A. **94**, 4330–4335
34 Inoki, K., Li, Y., Zhu, T., Wu, J. and Guan, K. L. (2002) TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat. Cell Biol. **4**, 648–657
35 Arcaro, A., Volinia, S., Zvelebil, M. J., Stein, R., Watton, S. J., Layton, M. J., Gout, I., Ahmadi, K., Downward, J. and Waterfield, M. D. (1998) Human phosphoinositide 3-kinase C2beta, the role of calcium and the C2 domain in enzyme activity. J. Biol. Chem. **273**, 33082–33090
36 Fratti, R. A., Backer, J. M., Gruenberg, J., Corvera, S. and Deretic, V. (2001) Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. J. Cell Biol. **154**, 631–644
37 Franke, T. F., Kaplan, D. R., Cantley, L. C. and Toker, A. (1997) Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science **275**, 665–668
38 Morris, M. C., Depollier, J., Mery, J., Heitz, F. and Divita, G. (2001) A peptide carrier for the delivery of biologically active proteins into mammalian cells. Nat. Biotechnol. **19**, 1173–1176
39 Patti, M. E., Brambilla, E., Luzi, L., Landaker, E. J. and Kahn, C. R. (1998) Bidirectional modulation of insulin action by amino acids. J. Clin. Invest. **101**, 1519–1529
40 Peyrollier, K., Hajduch, E., Blair, A. S., Hyde, R. and Hundal, H. S. (2000) L-leucine availability regulates phosphatidylinositol 3-kinase, p70 S6 kinase and glycogen synthase kinase-3 activity in L6 muscle cells: evidence for the involvement of the mammalian target of rapamycin (mTOR) pathway in the L-leucine-induced up-regulation of system A amino acid transport. Biochem. J. **350**, 361–368
41 Brunn, G. J., Williams, J., Sabers, C., Wiederrecht, G., Lawrence, Jr, J. C. and Abraham, R. T. (1996) Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. EMBO J. **15**, 5256–5267
42 Dennis, P. B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S. C. and Thomas, G. (2001) Mammalian TOR: a homeostatic ATP sensor. Science **294**, 1102–1105
43 Stenmark, H., Aasland, R. and Driscoll, P. C. (2002) The phosphatidylinositol 3-phosphate-binding FYVE finger. FEBS Lett. **513**, 77–84
44 Panaretou, C., Domin, J., Cockcroft, S. and Waterfield, M. D. (1997) Characterization of p150, an adaptor protein for the human phosphatidylinositol (PtdIns) 3-kinase. Substrate presentation by phosphatidylinositol transfer protein to the p150.Ptdins 3-kinase complex. J. Biol. Chem. **272**, 2477–2485
45 Deval, C., Mordier, S., Obled, C., Bechet, D., Combaret, L., Attaix, D. and Ferrara, M. (2001) Identification of cathepsin L as a differentially expressed message associated with muscle wasting. Biochem. J. **360**, 143–150
46 Auranen, M., Villanova, M., Muntoni, F., Fardeau, M., Scherer, S. W., Kalino, H. and Minassian, B. A. (2000) X-linked vacuolar myopathies: two separate loci and refined genetic mapping. Ann. Neurol. **47**, 666–669
47 Tanaka, Y., Guhde, G., Suter, A., Eskelinen, E. L., Hartmann, D., Lullmann-Rauch, R., Janssen, P. M., Blanz, J., von Figura, K. and Saftig, P. (2000) Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. Nature (London) **406**, 902–906
48 Larsen, K. E. and Sulzer, D. (2002) Autophagy in neurons: a review. Histol. Histopathol. **17**, 897–908
49 Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H. and Levine, B. (1999) Induction of autophagy and inhibition of tumorigenesis by Beclin 1. Nature (London) **402**, 672–676
50 Donati, A., Cavallini, G., Paradiso, C., Vittorini, S., Pollera, M., Gori, Z. and Bergamini, E. (2001) Age-related changes in the autophagic proteolysis of rat isolated liver cells: effects of antiaging dietary restrictions. J. Gerontol. A Biol. Sci. Med. Sci. **56**, B375–B383

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